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[CONTRIBUTIONS FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF ILLINOIS.]

THE CHEMISTRY OF FLESH.

(FOURTH PAPER.)[1]

A STUDY OF THE PROTEIDS OF BEEF FLESH.[2]

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THE past researches upon the proteids of animal substances have been mainly devoted to the study of the proteids of blood and to the proteids of muscle freed from blood. However, the flesh of the lower animals, as it is sold for food, is a mixture of muscle and blood, each of which is a very complex substance composed of many chemical bodies in varying proportions.

Notwithstanding the large amount of valuable research which has been devoted to the chemistry of the proteids of muscle and blood, the present knowledge of these substances is very incomplete, imperfect and contradictory. This is especially true of the chemistry of muscle. The literature of this subject is at present in a state of much confusion. Hammarsten,[3] the distinguished physiological chemist, in the last edition of his text-book in considering the proteids of muscle, says: "The views of the various investigators differ so essentially and the nomenclature is so complicated that it is extremely difficult to give any correct review of the various notions. For these reasons the author is not sure whether he has understood and correctly given

[1] This Journal, 26, 1086 (1904); 27, 658 (1905); 28, 25 (1906).

[2] This research was made possible by a grant from the Elizabeth Thompson Science Fund. We wish hereby gratefully to acknowledge the aid thus received in our investigations.

[3] "Text-book of Physiological Chemistry," 4th English Ed. (1904), p. 382.

the work of the different investigators. Thorough investigations on this subject are very necessary."

If the above can be said regarding the present knowledge of the proteids of muscle, it is quite evident that little is known of the chemistry of the proteids of flesh since in the first place it is a complex mixture and in the second place it has been studied but little. From the standpoint of physiological chemistry, it is highly desirable that the present very limited knowledge of the proteid substances of flesh be increased.

In a former paper[1] from this laboratory the results of the study of the solubility of the different nitrogenous constituents of flesh were given. The data thus reported were obtained by extracting flesh successively with the following reagents: Cold water, 10 per cent. sodium chloride solution, 0.15 per cent. hydrochloric acid solution, 0.15 per cent. potassium hydroxide solution and lastly with hot water. The results of the above preliminary work, together with other results obtained in later work, led to the conclusion that much additional information regarding the proteids of flesh could be obtained by extracting flesh successively with cold water, with a 10 per cent. ammonium sulphate solution, and finally with N/20 potassium hydroxide solution in the cold.

In presenting the results of the present investigation the subject may be most conveniently discussed under the following heads: A, acidity of flesh; B, proteids of flesh soluble in cold water; C, proteids of flesh insoluble in cold water but soluble in a 10 per cent. solution of ammonium sulphate; and D, proteids of flesh insoluble in cold water and in a 10 per cent. solution of ammonium sulphate but soluble in N/20 potassium hydroxide solution in the cold.

A. THE ACIDITY OF FLESH.

In order to study in detail the chemistry of the proteids of flesh, it was deemed necessary in the first place to investigate the nature of the acidity of flesh. There are at least three different classes of chemical substances which produce the acidity of flesh. They are acid phosphates, organic acids and proteids. The acid phosphates and the organic acids of flesh are mainly soluble in water while the greater portion of the proteids of flesh are insoluble in this solvent. The two chief acid phosphates

[1] This Journal, 26, 1086 (1904).

existing in the water extracts of meat are potassium dihydrogen phosphate and potassium monohydrogen phosphate. In fact these two salts are the chief mineral constituents existing in such solutions. Fractional precipitation and recrystallization of the constituents of aqueous extracts of flesh which have been made in connection with these studies have led to the conclusion that potassium dihydrogen phosphate is the acid phosphate which predominates. Potassium dihydrogen phosphate is acid to phenolphthalein and potassium monohydrogen phosphate is neutral to this indicator.

The chief organic acid occurring in flesh is lactic, which is readily soluble in water. Other soluble or insoluble organic acids exist only in very small quantities in flesh. The only indicator which at present is known to give good results with lactic acid is phenolphthalein. All other indicators are considered useless for titrating this acid.

The fact that proteids combine with bases is well-known. Osborne[1] has shown that the proteid bodies, as hitherto prepared, are in fact, definite chemical compounds of proteid substances with common mineral acids, or contain such compounds in admixture. To render such compounds neutral to phenolphthalein requires the addition of a notable quantity of an alkaline solution. The addition of the base removes the acid in combination with the proteid. If the proteids occurring in flesh are in combination with acids they will therefore probably react acid to phenolphthalein. Investigations made in this laboratory confirm this conclusion since all the proteid preparations which have so far been obtained from flesh, regardless of the method of preparation, react acid to phenolphthalein. Even the coagulated proteids separated from the aqueous extracts by continued heating in neutral solutions behave in this manner. It is thus evident that the soluble and insoluble proteids of flesh will have to be taken into account in determining the acidity of the same.

As yet methods for accurately determining the acidity due to each of these classes of substances, namely, acid phosphate, organic acids and proteids, have not been perfected. The results so far obtained indicate that it will be possible to accomplish this end in the near future.

[1] This Journal, 21, 486 (1899); 24, 39 (1902).

Acidity of the Cold Water Extracts of Flesh.—At present it is possible to determine with accuracy the total acidity of extracts of flesh when phenolphthalein is used as the indicator. Water extracts of flesh are slightly acid to litmus and azolitmin but the end reactions with these indicators in such solutions are very indefinite and unsatisfactory. The aqueous extracts are alkaline to methyl orange and neutral to cochineal; however, the former indicator would not give even a fairly sharp end reaction upon titration. None of the following indicators, rosolic acid, Congo red, lacmoid, or Bismark brown could be used on account of the very indefinite end reactions which they gave.

Preparation of the Cold Water Extracts.—Lean beef round from which all visible fat and connective tissue were removed has been used in the following work: The samples were thoroughly ground in a meat chopper and intimately mixed. For the work here reported the water extracts were prepared as follows: The finely divided meat was digested for one hour with ice water in the ratio of 1000 grams of meat to 1500 cc. of water. The resulting solution was filtered through fine cheese-cloth, the process being assisted by squeezing the cloth and its contents with the hand. The residue thus obtained was divided into smaller portions, and washed successively in series, using fresh water on number one only, until all soluble proteid was removed. Each washing was filtered through cheese-cloth as above. This operation was continued until the filtered extracts were colorless, and neutral to phenolphthalein and until they gave no reaction for proteid by the biuret test. The completely extracted residue had a grayish white color. A complete removal of the water-soluble constituents was obtained by using about 5000 cc. of water for each 1000 grams of flesh. The mixed filtrates and washings readily filtered through paper, giving a perfectly clear red filtrate.

The following table gives the results obtained in the determination of the acidity of the aqueous extracts of a number of samples of raw beef. The indicator used in the titrations was phenolphthalein.

TABLE I.—ACIDITY OF AQUEOUS EXTRACTS OF RAW BEEF FLESH.

Laboratory No.	Kind of meat.	Fresh substance. Total weight of meat taken. Grams.	Fresh substance. Total volume of aqueous extract. cc.	Fresh substance. Volume of N/10 alkali required for 100 cc. of extract. cc.	Fresh substance. Volume of N/10 alkali required for 100 grams of flesh. cc.	Fresh substance. Acidity calculated as lactic acid. Per cent.	Water-free substance. Water-free substance. Per cent.	Water-free substance. Acidity calculated as lactic acid. Per ct.
306	Beef round, lean ...	1699.000	10,000	16.54	97.34	0.88	24.31	3.62
211	Beef round, lean ...	4365.800	20,000	15.58	73.78	0.66	28.92	2.28
302	Beef round, lean ...	1585.500	11,000	12.19	84.53	0.76	26.30	2.89
1265	Beef round, lean ...	100.000	2,450	4.86	119.00	1.07	22.50	4.76
1266	Beef round, lean ...	100.000	2,500	4.22	105.60	0.95	25.28	3.76
1906	Beef round, lean ...	101.532	5,000	1.64	80.76	0.73	26.22	2.78
1916	Beef round, lean ...	99.536	5,000	2.00	100.46	0.90	26.04	3.46
	Average	.....	.....	.....	.....	0.85	.....	3.36

The total acidity of aqueous extracts of flesh varies between comparatively wide limits. The acidity of the extracts of lean beef round calculated as lactic acid and expressed in terms of the fresh substance of the meat varies from 0.66 to 1.07 per cent. This variation in the degree of acidity is probably due to the length of time, and the temperature at which the flesh has been kept from the time of the slaughtering of the animal until it is examined in the laboratory. This phase of the subject is now being studied.

Data taken from unpublished manuscript of this laboratory demonstrate the fact that the average amount of soluble inorganic phosphorus in fresh lean beef round is equal to 0.12 per cent. Assuming that all of this soluble inorganic phosphorus exists in the form of potassium dihydrogen phosphate (KH_2PO_4) there would be 0.526 per cent. of this salt. The titration of this quantity of potassium dihydrogen phosphate with a standard alkali solution using phenolphthalein as an indicator would represent a maximum acidity due to acid phosphates of 0.35 per cent. calculated as lactic acid. This calculated acidity due to acid phosphates is without doubt higher than it really should be, for it is a well-known fact that a certain, as yet unknown, proportion of the soluble inorganic phosphorus of the cold water extracts of flesh exists as dimetallic monohydrogen phosphates, *e. g.*, K_2HPO_4, which are neutral to phenolphthalein.

However, assuming for the time being that the maximum acidity due to acid phosphates may reach as much as 0.35 per

cent. calculated as lactic acid, then the average acidity of cold water extracts of flesh which is due to organic acids, proteids and other substances is at least 0.50 per cent. calculated to the same basis. How much of this latter acidity is due to each of the above-mentioned classes of substances is at present unknown, but experimental researches are now under way in this laboratory which will undoubtedly throw some light upon this problem.

Acidity of Meat Residues Insoluble in Cold Water.—The residues of meat which were left after the complete extraction with cold water were distinctly acid to litmus and to phenolphthalein, notwithstanding the fact that the last portions of the cold water extracts were entirely neutral to phenolphthalein. It has been found very difficult to determine directly the acidity of these residues because a satisfactory end reaction could not be obtained. At first the color produced by the alkali solution in the presence of phenolphthalein disappeared very readily, but towards the end of the titration the color leaves the solution slowly and much less readily. However, by suspending weighed quantities of the proteids in water, in glass-stoppered cylinders so that thorough stirring and intimate contact followed, approximately accurate results were obtainable.

Proceeding in this way a sample of completely extracted residue of beef round weighing 18.3744 grams, required 6.95 cc. of N/10 potassium hydroxide for neutralization. This is equivalent to 0.34 per cent. acidity calculated as lactic acid. The extracted residue contained 23.46 per cent. of water-free substance so that the acidity upon the water-free basis amounted to 1.45 per cent. calculated as lactic acid.

Again, a second sample of another completely extracted residue of beef round weighing 10.00 grams required 5.06 cc. of N/10 potassium hydroxide for neutralization. This is equivalent to 0.46 per cent. acidity calculated as lactic acid. In this case the extracted residue contained 30.74 per cent. of water-free substance so that the acidity as lactic acid calculated to the water-free basis equaled 1.49 per cent.

The difficulty which was experienced in obtaining a satisfactory end reaction in determining the acidity of these residues by direct titration led to an attempt to determine this quantity by indirect titration, that is, the added excess of the standard alkali was determined by titrating back with standard acid. In this

TABLE II.—ACIDITY OF INSOLUBLE MEAT RESIDUES—DETERMINED BY TREATING WITH EXCESS OF N/10 ALKALI AND THEN TITRATING BACK WITH N/10 ACID.

Laboratory No.	Kind of meat.	Fresh substance. Weight of residue. Grams.	Fresh substance. Volume of N/10 KOH added in excess for each 100 grams of residue. cc.	Fresh substance. Time of digestion. Hours.	Fresh substance. Volume of N/10 HCl required to produce neutralization. cc.	Fresh substance. Volume of N/10 KOH combined with 100 grams of residue. cc.	Fresh substance. Acidity calculated as lactic acid. Per cent.	Water-free substance. Water-free substance. Per cent.	Water-free substance. Acidity calculated as lactic acid. Per cent.	Remarks.
203	Beef round....	18.3794	126.7	3–4	62.6	64.1	0.58	23.46	2.47	Not filtered.
301*a*	Beef round....	8.4273	1379.5	20	1065.0	314.5	2.83	23.89	11.85	Filtered.
301*b*	Beef round....	5.1906	2239.6	20	1921.6	318.0	2.86	23.89	11.97	Filtered.
307*a*	Beef round....	3.7455	372.4	3–4	233.4	139 0	1.25	....	....	Not filtered.
307*b*	Beef round....	3.0626	455.7	3–4	297.6	158.1	1.42	....	....	Not filtered.
307*c*	Beef round....	3.0702	454.6	3–4	287.8	166.8	1.50	....	....	Not filtered.
320*a*	Beef round....	10.0000	139.1	About 2	55.6	83.5	0.75	30.74	2.47	Not filtered. Clear supernatant liquid was used.
320*b*	Beef round....	10.0000	139.1	About 2	58.4	80.7	0.73	30.74	2.37	Not filtered. Clear supernatant liquid was used.
320*c*	Beef round....	10.0000	139.1	About 18	53.8	85.3	0.77	30.74	2.50	Not filtered. Clear supernatant liquid was used.
301*c*[1]	Beef round....	10.0000	2325.0	Several. Filtered over night.	1159.4	1165.6	10.49	91.96	11.47	Filtered.
301*d*[1]	Beef round....	1.3443	1729.5	Several hours.	1397.4	332.1	2.99	91.96	3.25	Not filtered.

[1] Fresh meat residues were used in all experiments except the last two, in which the samples were air-dried after washing with alcohol and ether.

way the end reaction was quite sharp and distinct, but quite unexpected results were obtained as will be seen from the analytical data given in Table II and the discussion which follows.

It is quite evident from the results given in the above table that the residues of flesh which are insoluble in cold water, when treated with excess of dilute alkali solutions behave as acids in that they neutralize alkalies. By this action a considerable proportion, in some cases practically all of the residue, is dissolved by the alkali. The insoluble residues after air-drying by the use of alcohol and ether still show the same reactivity towards dilute alkali solutions. The extent of the action varies much but seems to be dependent primarily upon the strength of the alkaline solution and upon the length of the time of action. The insoluble residues consist almost entirely of proteid matter and the neutralizing action of the same is undoubtedly due to the proteids.

The clearly filtered, dilute alkaline solutions obtained in the above manner remain clear upon neutralization and in order to cause beginning precipitation the solution must be decidedly acid. In order to show clearly the nature of these solutions of the insoluble residues there is given below in detail the results of one experiment in this connection. The moist meat residue, No. 301, which had been thoroughly washed with cold water until free from all soluble material, was distinctly acid to litmus and to phenolphthalein. The moist residue amounted to 63.00 per cent. of the original fresh meat. Duplicate samples of the same, which were dried at a temperature of 104° until constant in weight, gave as an average result 23.89 per cent. of water-free substance.

Samples of the moist residue, weighing A, 8.4273 grams and B, 5.1906 grams, were transferred to 250 cc. flasks and after moistening thoroughly with water they were each treated with 116.3 cc. of N/10 KOH solution. The samples thus treated were allowed to stand for twenty hours with frequent shaking, diluted to exactly 250 cc., thoroughly mixed and filtered through dry filters.

The residues were almost completely dissolved by this treatment. Ten cc. portions of the filtrates were titrated to neutrality to phenolphthalein with N/10 HCl, with the following average results: A, 3.59 and B, 3.99 cc. For neutralization of the entire

sample A, 89.75 cc. and B, 99.75 cc. of N/10 HCl were required. As 116.3 cc. of N/10 KOH were added to each sample to begin with, the insoluble residues by digesting, at the ordinary temperature, with the dilute alkaline solution, neutralized the following quantities of N/10 KOH solution: A, 26.55 cc. and B, 16.55 cc. These results when calculated to 100 grams of the moist residue give the following figures: A, 315.0 cc.; B, 318.8 cc.; average, 316.9 cc. of N/10 KOH solution.

The neutralized solutions containing 10 cc. portions of the original 250 cc. alkaline filtrates thus obtained were entirely clear, and required in order to cause precipitation to begin, the following quantities of N/10 HCl solution in addition to that already added to produce neutralization: A, 0.62 cc. and B, 0.41 cc. Calculated to the entire samples these data give the following figures: A, 15.50 cc. and B, 10.25 cc. These results when calculated to 100 grams of the moist residue are equal to the following quantities: A, 183.9 cc.; B, 197.5 cc.; average, 190.7 cc. of N/10 HCl.

The addition of more acid until a certain definite amount had been added increased the precipitation of proteid matter, but when the volume of acid thus added was equal to the excess of alkali previously added no further precipitation resulted. However, all of the proteid is not in this way removed from the solution and the addition of acid beyond this point causes resolution of the precipitated proteid.

After the precipitation was made as complete as possible, there was required the following amount of N/10 HCl solution to completely redissolve it: A, 1.14 cc. and B, 0.83 cc. Calculated to the entire samples these data give the following figures: A, 28.50 cc. and B, 23.25 cc. In order to effect beginning precipitation in these acid solutions it required the addition of the following amounts of N/10 KOH solution, calculated to the whole samples: A, 15.35 cc. and B, 14.65 cc. Subtracting the latter amounts from those given immediately above there is obtained for A, 13.15 cc. and for B, 8.60 cc. which represents the amount of N/10 HCl solution necessary to hold the proteids in solution. These results when calculated to 100 grams of the moist residue give the following figures: A, 156.0 cc.; B, 165.6 cc.; average 160.8 cc. of N/10 HCl. The addition of slightly more alkali

causes precipitation but the addition of alkali beyond a certain fixed amount causes the proteid to redissolve.

It should also be mentioned in this connection that fresh flesh gives reactions with dilute alkali solutions which are similar to those described above for the insoluble residues left after completely extracting flesh with cold water. That this is true can be seen from the data presented in Table III.

Further, the insoluble proteids of flesh upon digestion with N/10 hydrochloric acid solution at the ordinary temperature combine with the latter. In one experiment 10 grams of an insoluble residue which had been air-dried after washing with alcohol and ether were treated with 189.0 cc. of N/10 hydrochloric acid solution for six hours. The solution was then made up to a definite volume and filtered through a dry filter. Aliquot portions of the filtrate were titrated with N/10 potassium hydroxide solution, using phenolphthalein as an indicator. Calculating the results to the entire 10 gram portion, 117.2 cc. of N/10 potassium hydroxide solution were required to bring about neutralization. That is to say, 68.8 cc. of the N/10 hydrochloric acid solution had combined with the 10 grams of the air-dried residue of flesh. The air-dried residue contained 8.04 per cent. of water.

A discussion of the above results of the action of bases and acids upon the different preparations obtained from flesh is at present withheld until further quantitative studies which are now in progress are completed and entirely available.

B. PROTEIDS OF FLESH SOLUBLE IN COLD WATER.

Previous investigations[1] in this laboratory have demonstrated the facts: (1) that a considerable proportion of raw flesh is soluble in cold water; (2) that the cold water extract of raw flesh contains a marked quantity of the total proteid of the flesh; (3) that the extraction of flesh with cold water may be made complete; (4) that the proteid material of the cold water extract of raw flesh consists chiefly of proteids which are coagulable by heat, but there is a small amount of proteid substances which do not coagulate but are precipitated by saturating their solution with zinc sulphate, and there is also apparently a very small amount of proteid matter which is neither coagulated by heat nor precipitated by zinc sulphate but which is precipitated by tannin and salt. Recently complete chemical analyses of the cold

[1] This Journal, 26, 1086 (1904); 27, 658 (1905).

TABLE III.—ACIDITY OF FRESH MEAT DETERMINED BY TREATING WITH EXCESS OF N/10 ALKALI AND THEN TITRATING BACK WITH N/10 ACID.

		Fresh substance.						Water-free substance.		
Laboratory No.	Kind of meat.	Weight of meat taken. Grams.	Volume of N/10 KOH added in excess for each 100 grams of meat. cc.	Time of digestion. Hours.	Volume of N/10 HCl required to produce neutralization. cc.	Volume of N/10 KOH combined with 100 grams of meat. cc.	Acidity calculated as lactic acid. Per cent.	Water-free substance. Per cent.	Acidity calculated as lactic acid. Per cent.	Remarks.
300*a*	Beef round.....	9.1986	1011.0	About 18	687.5	323.5	2.91	26.30	11.06	Filtered.
300*b*	Beef round.....	8.5638	1086.0	About 18	754.7	331.3	2.98	26.30	11.33	Filtered.
305*a*	Beef round.....	5.9293	253.0	3	112.9	122.5	1.10	24.31	4.52	Not filtered.
305*b*	Beef round.....	5.0645	275.5	3	148.7	126.8	1.14	24.31	4.69	Not filtered.
305*c*	Beef round.....	5.0113	278.3	3	154.0	124.3	1.12	24 31	4.61	Not filtered.
315*a*	Beef round.....	10.0000	139.1	About 18	9.3	129.8	1.17	30.73	3.81	Not filtered. Clear, supernatant liquid used.
315*b*	Beef round.....	10.0000	139.1	About 18	Alkali 13.9	153.0	1.38	30.73	4.41	Not filtered. Clear, supernatant liquid used.
315*c*	Beef round.....	10.0000	139.1	About 18	none	139.1	1.25	30.73	4.07	Not filtered. Clear, supernatant liquid used.

water extracts of 18 samples of lean beef round have been made in connection with these researches. The average percentage of water in these samples was 73.86. The average composition[1] of water extracts of these 18 samples of raw beef round expressed in percentage of the fresh substance is as follows: Total soluble dry substance 6.46 per cent., total soluble proteid 2.51 per cent., consisting of 2.26 per cent. coagulable proteid, 0.21 per cent. albumoses and 0.02 per cent. peptones, total organic extractives 3.04 per cent., consisting of 1.23 per cent. nitrogenous and 1.80 per cent. non-nitrogenous extractives and ash 0.92 per cent. The total nitrogen in these extracts amounted to 0.795 per cent., of which 0.401 per cent. existed as proteid and 0.394 per cent. as non-proteid nitrogen.

The average amounts of total proteid existing in these eighteen samples of lean beef round equaled 18.96 per cent. It is thus evident that 13.56 per cent. of the total proteid existing in lean beef is soluble in cold water. Of this total soluble proteid 90.04 per cent. is in a form which is coagulable by heat from a neutral solution, 8.40 per cent. exists as albumoses and a very small quantity apparently exists in the form of peptones. It is not supposed that the albumoses and peptones existed as such in the original flesh. It is more than probable that the substances which are represented in the above analyses as albumoses and peptones have been formed from the original proteids of the flesh by the treatment through which the latter have passed during their extraction, separation and estimation.

Fractional Coagulation by Heat of the Proteids of Flesh Soluble in Water.—The presence of a considerable quantity of proteid substances in the cold water extracts of flesh makes it desirable to determine their character. Fractional coagulation by heat has long been used as a means of separating different proteids existing in solution. Halliburton,[2] in studying the proteids of muscle plasma, made much use of this method of fractional coagulation in order to distinguish and also to separate the different proteids of this liquid. He gives very definite temperatures at which the different fractions separate and maintains that the proteids separating at the different temperatures are not identical.

[1] Data taken from unpublished manuscript of this laboratory.

[2] J. Physiol. 8, 133 (1887); Biochemistry of Muscle and Nerve (1904).

Upon the basis of Halliburton's work it seemed best in the first place to attempt the separation of the proteids of flesh soluble in cold water by means of fractional precipitation by heat. This was considered best notwithstanding the fact that we fully realized that in the water extracts of flesh we were dealing with solutions quite different in character from those of muscle plasma especially as regards concentration, acidity, amount of other substances present and the nature of the proteids which they contain.

The method used in preparing the water extracts for this part of the work was the same as that described above (page 472). In this connection, it should be said that if the concentrated extract did not filter rapidly, it was mixed with some of the washings which gave a dilution that filtered readily through filter-paper, giving a perfectly clear solution.

A number of preliminary experiments which it is not necessary to describe here in detail led to the following information regarding the fractional coagulation of aqueous extractions of flesh.

First, during coagulation there is an increase in the acidity of the solutions.

Second, by reducing the acidity of the aqueous extracts of flesh, the coagulation of the same takes place in better form and the filtration of the solution is much facilitated.

Third, the partial neutralization of the acidity of the aqueous extracts of flesh even at the ordinary temperature causes the precipitation of some of the proteid matter which they contain.

Fourth, the complete removal of the proteid coagulating at any fixed definite temperature requires long application of heat. Further, the lower the temperature at which a coagulum is separated the longer the time of heating required to effect complete coagulation.

Fifth, there are no well defined degrees of temperature at which different coagula of aqueous extracts of flesh separate. That this statement is true is shown by the following description of several experiments. About 75 cc. of a clear water extract of fresh meat were placed in a large bath of cold water which was very gradually heated. At a temperature of 41° an opalescence was first noted. At this point, the temperature was held stationary for fifteen minutes without apparent increase in the

opalescence. The temperature of the solution was gradually increased to 43° when the opalescence became more marked. There was however no separation of a coagulum. At 44° a fine precipitate began to separate. The solution was maintained at this temperature for one hour. A considerable quantity of a nearly white precipitate settled in a finely divided condition to the bottom of the vessel, but the solution did not become clear nor did the precipitate collect as a coagulum. Again, a one liter sample of the same extract was maintained at a temperature of 43° for two hours; a decided turbidity but no distinct coagulation resulted. Upon maintaining the temperature of the solution at 43° for twelve hours a distinct coagulum was obtained. The coagulum was removed by filtration and the perfectly clear filtrate resulting was again maintained at a temperature of 43° for twelve hours. A further coagulation resulted. This second coagulation was removed by filtration and the clear filtrate again subjected to a temperature of 43° for twelve hours. This time only a very small coagulum was formed. In a third test a 100 cc. sample of the same water extract as used in the above tests was kept at a temperature of 30° for five hours. No change was noted during the first two hours; then a precipitate began gradually to collect in the bottom of the flask so that at the end of the five hours there was quite an appreciable coagulum formed.

Sixth, the coagula are obtained in loose flocculent powders, if washed thoroughly, first with water, then with strong alcohol and finally with ether and then constantly stirred while drying them free from ether. If the samples are prepared and dried in this manner, they never harden into cakes and they are very uniform in character.

Seventh, after a coagulum separating at a definite temperature is completely removed and the filtrate from the same remains perfectly clear after being heated for several hours at this fixed temperature, a slight increase of temperature of only 2° or 3° for a comparatively short time—twenty to thirty minutes—causes an additional coagulum to commence to form.

Although the results of these preliminary experiments, a summary of which is given above, indicated quite conclusively that fractional coagulation would not effect a sharp separation between the proteids of flesh soluble in water, it was determined

to investigate this subject further by separating several coagula from such solutions under known conditions and studying in detail their physical and chemical properties in order to prove definitely the differences between the proteids thus coagulated at various temperatures.

Composition of a Complete Coagulum of a Water Extract of Flesh.—A water extract was prepared from the lean of a sample of beef round taken from a fat, export steer. The method used in preparing the extract was the same as described above (page 472). A portion of the extract representing seven and one-half pounds of the meat was completely coagulated by heating it upon the water-bath until the solution had been reduced to one-fourth of the original volume.

The first coagulum which formed somewhat below 50° was nearly white, but as the temperature of the solution increased the color of the coagulum became darker while the supernatant liquid grew lighter in color, although it never became colorless.

The completely coagulated extract was filtered and thoroughly washed with boiling water. The dark brown coagulum was washed with alcohol and ether and then air-dried. This sample (laboratory No. 101) which represents the total coagulable proteid separated by heat from a water extract of lean meat was dried to constant weight at 104° and analyzed with the following average results: C, 51.71; H, 7.35; N, 15.85; S, 0.98; ash, 1.05.

The proportion of nitrogen in the different forms of combination that occur in the decomposition products produced by boiling the proteid with hydrochloric acid was determined by Hausmann's[1] method as modified by Osborne and Harris.[2]

The following average results were obtained:

TABLE IV.—PERCENTAGE OF NITROGEN IN THE DIFFERENT GROUPS IN THE COMPLETE COAGULUM OF A WATER EXTRACT OF FLESH.

	Per cent.
Nitrogen as ammonia	1.01
Basic nitrogen	4.49
Non-basic nitrogen	10.03
Nitrogen in magnesium oxide precipitate	0.32
Nitrogen in the humin substances	0.07
Total nitrogen by summation	15.92
Total nitrogen in proteid	15.85

[1] Z. physiol. Chem. 27, 92 (1899).

[2] This Journal, 25, 323 (1904).

Methods Used in Determining the Hydrolytic Products.—As a result of a somewhat extended study of the hydrolytic products resulting from the decomposition of the proteids of flesh with boiling hydrochloric acid, it seemed desirable to modify to a certain extent the Osborne and Harris modification of the Hausmann method. The method finally adopted for the work here reported is given herewith in detail. One gram portions of the proteid were hydrolyzed by boiling them in 500 cc. Kjeldahl flasks with 100 cc. of a 20 per cent. solution of hydrochloric acid for about ten hours. The excess of acid was removed by evaporating the solution to a volume of about 3 cc. Approximately 100 cc. of water were added to the concentrated liquid, which was then filtered and the precipitate thoroughly washed and returned to the Kjeldahl flask for the determination of the amount of nitrogen present in the humin substances. The combined filtrate and washings were carefully treated with magnesium oxide paste until distinctly alkaline to phenolphthalein and distilled for the nitrogen existing as ammonia. The distillation was continued until the volume in the nitrogen flask was about 50 cc. After cooling, the contents of the flask were filtered and thoroughly washed until the filtrate was neutral to phenolphthalein. The magnesium oxide precipitate was returned to the nitrogen flask and the amount of nitrogen determined by the Kjeldahl method.

TABLE V.—NITROGEN GIVEN OFF AS AMMONIA DURING THE CONCENTRATION OF THE ALKALINE FILTRATE.

Laboratory No.	Nitrogen as ammonia. First distillation. Per cent.	Second distillation. Per cent.	Total. Per cent.
101*a*	0.96	0.04	1.00
101*b*	0.96	0.03	0.99
101*c*	0.97	0.05	1.02
101*d*	1.00	none	1.00
101*e*	0.99	0.03	1.02
307*a*	1.00	0.08	1.08
307*b*	1.08	0.01	1.09
302*a*	0.76	0.02	0.78
302*b*	0.74	0.05	0.79
110*a*	1.14	0.06	1.20
110*b*	1.15	0.07	1.22
110*c*	1.19	0.04	1.23

The filtrate and washings from the magnesium oxide precipitate were distilled to about 100 cc. and the ammonia in the distillate determined and added to the amount found in the first distillation, the total nitrogen thus obtained being reported as "Nitrogen as Ammonia." That this modification was necessary is shown by the data given in Table V.

The concentrated filtrate from the magnesium oxide precipitate was diluted to a volume of 200 cc., and 100 cc. of this solution, representing 0.5 gram of the proteid were acidified with sulphuric acid and then precipitated with 30 cc. of phosphotungstic acid as directed by Osborne and Harris. The combined filtrate and washings from the phosphotungstic acid precipitate were diluted to 500 cc. and the nitrogen determined in 100 cc. portions. As a check on these determinations the total nitrogen was determined in 50 cc. portions of the filtrate from the magnesium oxide precipitate. The character of the results thus obtained may be judged from the data given in the following table:

TABLE VI.—RESULTS ILLUSTRATING THE METHOD USED IN CHECKING THE DETERMINATION OF THE BASIC AND NON-BASIC NITROGEN.

Laboratory No.	Nitrogen in phosphotungstic acid precipitate. Per cent.	Nitrogen in filtrate from phosphotungstic acid precipitate. Per ct.	Sum of the nitrogen in the two previous columns. Per cent.	Total nitrogen in the filtrate from MgO precipitate. Per cent.	Difference. Per cent.
101*a*	4.47	10.18	14.65	14.65	none
101*b*	4.52	9.87	14.39	14.48	0.09
115*a*	4.54	10.02	14.56	14.50	0.06
115*b*	4.34	9.81	14.15	14.34	0.19
110*a*	4.49	8.50	12.99	13.53	0.54
110*b*	4.48	8.70	13.18	13.51	0.33

Composition and Properties of Fractional Coagula of Water Extracts of Flesh.—For this purpose a water extract was prepared as previously described from 1587 grams of selected, lean, beef round taken from a prime export steer. The water-free substance of this sample of meat equaled 26.30 per cent. of the fresh substance.

Eleven liters of the clear filtered extract were obtained which had a total acidity equivalent to 0.75 per cent. calculated as lactic acid. The acidity of this entire extract was reduced to 0.34 per cent. upon the same basis by the addition of the required

quantity of a standard solution of potassium hydroxide. The partial neutralization of the extract produced a considerable quantity of a precipitate which was nearly white in color. It was removed by filtration and thoroughly washed first with water, then with strong alcohol and finally with ether. The residue thus obtained was rubbed through a sieve while drying it from the ether and it was then given laboratory No. 302*a*. It was dried to constant weight at a temperature of 104°. The residue thus dried weighed 3.8071 grams and contained 16.44 per cent. of nitrogen.

The filtrate from the above precipitate which was produced by neutralization was heated for two hours at a constant temperature of 50°. A considerable amount of a nearly white coagulum was formed which was removed by filtration and thoroughly washed with water, alcohol and ether. The coagulum thus obtained, which was given laboratory No. 302*b*, weighed 7.6705 grams after drying to constant weight at 104° and contained 16.31 per cent. of nitrogen.

The filtrate from the above coagulum No. 302*b* was again heated for two hours at 50°. An additional coagulum which was almost white was obtained and after removal by filtration was thoroughly washed as above. It was given laboratory No. 302*c*. The proteid thus obtained weighed 3.6860 grams after it had been dried to constant weight at 104° and in this condition it contained 16.05 per cent. of nitrogen.

The solution from the second coagulum at 50° was again heated for two hours at this temperature. The nearly white coagulum which resulted was separated by filtration and thoroughly washed with water, alcohol and ether. The quantity of proteid thus obtained equaled 3.9308 grams after drying to constant weight at 104°. It contained 16.46 per cent. of nitrogen, and was labeled 302*d*.

The three fractions Nos. 302*b*, 302*c* and 302*d* of the coagulum occurring at 50° were made into a composite sample for the purpose of studying the hydrolytic products produced by boiling with hydrochloric acid

Hausmann's method as modified by Osborne and Harris gave the following results for the proportion of nitrogen in the different forms of combination occurring in the decomposition products.

TABLE VII.—PERCENTAGE OF NITROGEN IN THE DIFFERENT GROUPS IN THE PROTEID OF A WATER EXTRACT OF FLESH COAGULATING AT 50°.

	Per cent.
Nitrogen as ammonia	1.05
Basic nitrogen	4.44
Non-basic nitrogen	10.48
Nitrogen in magnesium oxide precipitate	0.33[1]
Nitrogen in the humin substances	.....
Total nitrogen by summation	16.30
Total nitrogen in proteid	16.27

By the coagulation of the three fractions Nos. 302*b*, *c*, *d*, of the proteid at a temperature of 50° the acidity of the solution had increased 80 per cent. The acidity of the entire extract was again reduced to 0.34 per cent. calculated as lactic acid, by the addition of a standard solution of potassium hydroxide. The neutralization produced a light gray precipitate which was separated from the solution by filtration, and then thoroughly washed with water, alcohol and ether. The precipitate which was labeled 302*e*, weighed 1.3058 grams after drying at 104° and contained 16.09 per cent. of nitrogen.

The filtrate from the above precipitation which was produced by neutralization was heated for four hours at a constant temperature of 50°. A further coagulum of a pinkish white color was obtained which was labeled 302*f*. It weighed 1.400 grams after it had been dried to constant weight at 104° and an analysis showed that it contained 15.62 per cent. of nitrogen. Mention should be made of the low content of nitrogen in this sample notwithstanding the fact that the nitrogen in the precipitate obtained by partial neutralization is about as high as it is in the preceding coagula. This agrees with results obtained in several other cases; namely, that heating following neutralization or partial neutralization gives a coagulum of less nitrogen content than the precipitate produced by the addition of the alkali. Further study of this problem is now being made.

The filtrate from the last coagulum was heated for four hours at a temperature of 75°, and as a result a large quantity of a grayish white coagulum was produced. After filtering, washing and drying as usual this fraction weighed 17.2103 grams and contained 16.54 per cent. nitrogen. It was given laboratory

[1] Includes the nitrogen contained in the humin substances.

No. 302*g*. This coagulum separating at 75° gave the following results by hydrolytic decomposition with hydrochloric acid.

TABLE VIII.—PERCENTAGE OF NITROGEN IN THE DIFFERENT GROUPS IN THE PROTEID OF A WATER EXTRACT OF FLESH COAGULATING AT 75°.

	Per cent.
Nitrogen as ammonia	1.00
Basic nitrogen	4.91
Non-basic nitrogen	9.80
Nitrogen in magnesium oxide precipitate	0.22
Nitrogen in the humin substances	0.05
Total nitrogen by summation	15.98
Total nitrogen in proteid	15.54

By heating the filtrate from 302*g* for four hours at 75°, a small quantity of a finely divided dark brown coagulum resulted. After separation and purification this coagulum weighed 0.9451 grams and by analysis it was found to contain 16.22 per cent. of nitrogen. This fractional coagulum was given laboratory No. 302*h*.

The filtrate from No. 302*h* was heated for eight hours at a temperature varying from 85° to 90°. A large quantity of a finely divided brick-red coagulum was formed which was removed by filtration and washed thoroughly with water, alcohol and ether. After drying at 104° this coagulum weighed 5.330 grams and contained 16.44 per cent. of nitrogen. It was labeled 302*i*.

This coagulum separating at 85° gave the following results by hydrolytic decomposition with hydrochloric acid.

TABLE IX.—PERCENTAGE OF NITROGEN IN THE DIFFERENT GROUPS IN THE PROTEID OF A WATER EXTRACT OF FLESH COAGULATING AT 85°.

	Per cent.
Nitrogen as ammonia	0.79
Basic nitrogen	4.11
Non-basic nitrogen	10.94
Nitrogen in magnesium oxide precipitate	0.53
Nitrogen in the humin substances	0.12
Total nitrogen by summation	16.49
Total nitrogen in proteid	16.44

The filtrate from the last precipitate was now exactly and completely neutralized with a standard solution of potassium hydroxide. No precipitation was noticeable at first but upon

TABLE X.—SUMMARY OF THE ABOVE STUDY OF FRACTIONAL COAGULATION.

Laboratory No.	Condition of the formation of the precipitates in the filtrate from the preceding precipitate.	Appearance of precipitates.	Weight of precipitates. Grams.	Nitrogen contained in the precipitates. Per cent.	Nitrogen factor.	Per cent. of each precipitate of the total weight of precipitates.	Per cent. of each precipitate calculated to the air-dried sample of meat.	Results of hydrolysis. Nitrogen as ammonia. Per cent.	Basic nitrogen. Per cent.	Non-basic nitrogen. Per cent.	Nitrogen in magnesium oxide precipitate. Per cent.	Nitrogen in the humin substances. Per cent.	Total nitrogen by summation. Per cent.	Nitrogen in the proteid. Per cent.
302*a*	By partial neutralization.....	Nearly white.	3.8071	16.44	6.11	8.37	0.91	...	...	....	...	...	....	....
302*b*	By coagulation, at 50°, for 2 hours	Nearly white.	7.6705	16.31	6.10	16.86	1.83							
302*c*	By coagulation, at 50°, for 2 hours	Nearly white.	3.6860	16.05	6.23	8.10	0.88	1.05	4.44	10.48	0.33[1]	...	16.30	16.27
302*d*	By coagulation, at 50°, for 2 hours	Nearly white.	3.9308	16.46	6.08	8.64	0.94							
302*e*	By partial neutralization again..	Light gray.	1.3058	16.09	6.22	2.87	0.31	...	...	....	...	...	....	....
302*f*	By coagulation, at 50°, for 4 hours	Pinkish white.	1.4000	15.62	6.40	3.08	0.36	...	...	....	...	...	....	....
302*g*	By coagulation, at 75°, for 4 hours	Gray-white.	17.2103	16.54	6.04	37.82	4.12	1.00	4.91	9.80	0.22	0.05	15.98	16.54
302*h*	By coagulation, at 75°, for 4 hours	Dark brown.	0.9451	16.22	6.09	2.08	0.23	...	...	....	...	...	....	....
302*i*	By coagulation, at 85°–90°, for 8 hours.....	Brick-red.	5.3320	16.44	6.08	11.72	1.27	0.79	4.11	10.94	0.53	0.12	16.49	16.44
302*j*	By complete neutralization and heat.....	Gray-white.	0.2160	2.91	...	0.47	0.05	...	...	....	...	...	....	....
			45.5036	16.24	6.10[2]	100.00	10.97	...	...	....	...	...	....	....

[1] Contains also the nitrogen of the humin substances.

[2] Taking into consideration the relative amounts of precipitates.

heating on the water-bath a small quantity of a light colored flocculent precipitate resulted which was removed by filtration and thoroughly washed in the usual manner. After drying this precipitate to constant weight at 104° it weighed 0.2160 gram and contained 2.91 per cent. of nitrogen. It was given laboratory No. 302*j*.

The conclusions to be drawn from the results of the experiments described in this connection are as follows:

First, by a study of the data presented in the above tables it will be found that the total proteid matter separated by neutralization and coagulation amounted to 2.86 per cent. of the weight of the flesh taken for the experiment. The proteids precipitated by neutralization formed 0.34 per cent., the proteids separated by coagulation at a temperature of 50° formed 1.05 per cent., while the proteids coagulating at 75°, 1.14 per cent. and the proteids coagulating at 85° formed 0.34 per cent. of the weight of the flesh taken for the study.

Expressed in another way, of the total proteid material of a cold water extract of raw flesh that is precipitated by neutralization and coagulation, 11.71 per cent. is separated by neutralization, 36.65 per cent. is precipitated by coagulation at a temperature of 50°, 39.93 per cent. coagulates at a temperature of 75° and 11.71 per cent. is precipitated by coagulation at a temperature of 85°.

Second, it is apparent that the chemical composition of the different coagula, at least so far as nitrogen content is concerned, is remarkably constant. The nitrogen in the several fractions varies from 15.62 to 16.54 per cent. not including the fraction 302*j* which was proved to be composed chiefly of calcium phosphate. The carbon, hydrogen, sulphur, phosphorus and ash contained in these coagula will be determined.

Third, it is evident that the results of the hydrolysis of the several fractions of the proteids coagulating at 50°, 75° and 85° indicate clearly that they are quite similar as regards their chemical constitution. Further study of the hydrolytic products of these different coagula is under way.

Fourth, the coagula obtained at temperatures of 48° to 50° from aqueous extracts of flesh are soluble in very dilute solutions of potassium and sodium hydroxides. They are reprecipitated

upon slightly acidifying with very dilute hydrochloric acid but soluble in the latter acid upon the addition of a very slight excess. This latter solution in hydrochloric acid is precipitated by the addition of sodium chloride to somewhat less than one-fourth saturation. The precipitate so formed is soluble in water and can again be reprecipitated from solution by the addition of sodium chloride.

The coagula obtained at 75° and at 85° from aqueous extracts of flesh are both soluble in a 0.5 per cent. solution of potassium hydroxide, forming a greenish brown solution. They are not as readily soluble in alkaline solutions as are the coagula formed at 50°.

A STUDY OF THE INFLUENCE OF ACIDITY UPON FRACTIONAL COAGULATION OF WATER EXTRACTS OF FLESH.

Previous work[1] done in this laboratory, together with the preliminary experiments which were made in connection with the research here reported, indicated very clearly that there is a close relationship existing between the act of the coagulation of the proteids and the acidity of the water extracts of flesh. In order to determine exactly these conditions as regards the degree of acidity best suited to effect the fractional coagulation of the proteids of aqueous extracts of flesh the following experiment was undertaken.

A water extract was prepared by the usual method from 1701 grams of lean beef round taken from a three-year-old heifer. Of the 10 liters of the extract which were prepared, 4 liters of the first and most concentrated portion were taken for the experiment here described. One hundred cubic centimeters of this extract contained 1.8964 grams of total solids dried at 104° and 0.2498 gram of nitrogen. The nitrogen in the extract therefore formed 13.18 per cent. of the total dry substance of the same. The total acidity of the entire extract calculated as lactic acid was equivalent to 1.18 per cent. of the fresh substance. Phenolphthalein was used as the indicator.

From this water extract five series of samples each measuring 200 cc. were taken. The samples were transferred to 500 cc. Kjeldahl flasks and treated as follows:

[1] This Journal, 27, 668 (1905).

(A) Triplicate samples of 200 cc. each. The acidity of the original extract was not changed. Acidity equaled 1.18 per cent.

(B) Triplicate samples of 200 cc. each. The acidity of the extract was reduced one-fourth. Acidity equaled 0.88 per cent.

(C) Triplicate samples of 200 cc. each. The acidity of the original extract was reduced one-half. Acidity equaled 0.59 per cent.

(D) Triplicate samples of 200 cc. each. The acidity of the original extract was reduced three-fourths. Acidity equaled 0.30 per cent.

(E) Triplicate samples of 200 cc. each. The acidity of the original extract was completely neutralized. The reaction of these solutions was neutral to phenolphthalein.

Upon adding the necessary amounts of a standard solution of potassium hydroxide to effect the neutralization above mentioned the following results were produced. In the B's there were only faint precipitates which were easily and rapidly filtered from the solutions.

In the C's there were small precipitates which collected into masses resembling coagula. These precipitates were easily and rapidly removed by filtration.

In the D's there were large quantities of precipitates which were separated fairly well by filtration but not so readily as those from B and C.

In the E's the precipitates produced by neutralization were large but they were separated only tardily by filtration.

The precipitates produced above were washed with water and the filtrates and washings collected in Kjeldahl flasks. The washed precipitates were returned to the flasks in which they had been precipitated and the nitrogen which they contained determined by the Kjeldahl method.

The filtrates from the above precipitates, together with solutions A to which no potassium hydroxide solution had been added, were all subjected to the following series of treatments. After each treatment the precipitates were separated by filtration and washed and then the nitrogen determined in them. The averages of the analytical results are given in Table XI.

First, they were heated for two hours at 50°. The triplicate samples of A gave turbid solutions which required twelve hours to filter and wash. The solutions B, C, and D all filtered rapidly, giving clear filtrates. Solution E filtered slowly but gave a clear filtrate.

Second, they were again heated for two hours at 50°.

Third, the solutions were again heated for two hours at 50°.

Fourth, they were heated at 50° for four hours.

Fifth, the solutions were heated at 50° for five and one-half hours.

Sixth, the solutions were then heated at 60° for two hours.

Seventh, they were heated at 60° for four hours.

Eighth, the solutions were further heated at 60° for four and one-half hours.

Ninth, they were heated at 70° for three hours.

Tenth, the solutions were further heated at 70° for three hours but as no precipitation occurred the temperature was increased to 80°. It required one hour for the temperature of the solutions to reach 80°. They were then kept at 80° for three hours.

Eleventh, the filtrates from the above were placed in a bath of boiling water. They became turbid in a few minutes. The solutions were maintained at this temperature (96°) for three hours.

Twelfth, the filtrates were all concentrated on the water-bath to a volume of about 50 cc. and the resulting precipitates removed by filtration.

Thirteenth, the filtrates and washings from the above were diluted to definite volume and an aliquot part taken for the determination of albumoses.

Fourteenth, a second aliquot part of the above solution was used for the determination of the peptones.

Fifteenth, a third aliquot portion of the solution was used for the determination of the total nitrogen so that from the data obtained under the last two headings the quantity of the nitrogenous extractives could be estimated.

A condensed summary of the analytical results of this experiment is given in the following table:

TABLE XI.—SUMMARY SHOWING THE INFLUENCE OF THE DEGREE OF ACIDITY UPON THE FRACTIONAL COAGULATION OF A COLD WATER EXTRACT OF FLESH.

(Results expressed in grams of nitrogen contained in total extract, 4000 cc.)

Nitrogen obtained.	A. Without neutralization. Grams.	B. One-fourth neutralization. Grams.	C. One-half neutralization. Grams.	D. Three-fourths neutralization. Grams.	E. Complete neutralization. Grams.	Time required for coagulation. Hours.
By addition of alkali	0.547[1]	0.099	0.240	0.469	0.514	..
By coagulation at 50°	2.768	2.497	2.277	2.478	2.718	15½
By coagulation at 60°	0.557	1.734	1.902	1.528	1.293	10½
By coagulation at 70°	0.293	0.553	0.404	0.258	0.250	7
By coagulation at 80°	0.992	0.721	0.873	0.794	0.660	3
By coagulation at 96°	0.066	0.233	0.162	0.106	0.108	39
Total by coagulation	4.677	5.738	5.618	5.165	5.030	..
By concentration	0.024	0.039	0.066	0.111	0.177	..
Albumoses and peptones	0.251	0.328	0.724	0.697	0.850	..
Extractives	3.530	3.778	3.547	3.504	3.383	..
Total nitrogen by summation	9.029	9.983	10.196	9.946	9.954	..

This experiment which was undertaken to determine the influence of acidity upon the fractional coagulation of water extracts of flesh leads to the following conclusions:

First, that it is necessary to effect at least partial neutralization of aqueous extracts of flesh in order that the different coagula may be of such a nature that they may be readily and completely removed by filtration from the liquids in which they were coagulated. In all cases, the extracts which had not been partially neutralized gave turbid solutions which required twelve to twenty-four hours for filtration and washing. In fact it was found to be impossible to complete successfully the above series of tests upon the solutions that had not been neutralized. That being the case after they had been heated to a temperature of 80° and the several fractions below this temperature had been with much difficulty incompletely removed it was found necessary to partially neutralize them before proceeding further with the experiment. As a result of this treatment the solutions which from the first had continually filtered very slowly, giving turbid filtrates, now filtered rapidly and gave perfectly clear filtrates.

Second, that one-fourth neutralization to phenolphthalein of the natural acidity of cold water extracts of flesh gave such con-

[1] In A, the neutralization was effected after heating to 80°.

ditions in the resulting solutions, that the separation of the coagulable proteids is more complete than it is if further neutralization of the acidity is produced. The total nitrogen in the combined fractions of the coagulated proteid in the 4 liters of extract in question for the different degrees of neutralization was as follows: One-fourth neutralization 5.738 grams, one-half neutralization 5.618 grams, three-fourths neutralization 5.165 grams and complete neutralization 5.030 grams. It is thus apparent that if the acidity of these solutions is decreased beyond that of one-fourth neutralization, there is a proportional decrease in the amount of proteids separated by coagulation.

Third, the data here presented point clearly to the conclusion that the more nearly the natural acidity of water extracts of flesh is neutralized before separating the coagulable proteids by the method here indicated, the greater is the quantity of proteids obtained as albumoses and peptones.

C. THE PROTEIDS OF FLESH INSOLUBLE IN COLD WATER BUT SOLUBLE IN A 10 PER CENT. SOLUTION OF AMMONIUM SULPHATE.

After the complete removal of the water-soluble constituents of flesh, a considerable portion of the residue was found to be soluble in a 10 per cent. solution of ammonium sulphate. Quantitative experiments have shown that a 10 per cent. solution of ammonium sulphate removes from the residue remaining after the complete extraction of flesh with water, 3.00 per cent. of proteid calculated upon the basis of the fresh substance of the flesh. The extraction of the residue insoluble in water, with the ammonium sulphate solution, was conducted in the same manner as the extraction of the original flesh with cold water. The extraction was continued until the last filtrate failed to give the biuret reaction for proteid. The ammonium sulphate extract after it was carefully strained through cheese-cloth without pressure, consisted of a turbid solution that could not be filtered through paper. By allowing this turbid solution to stand over night in cold storage, a gray-white flocculent precipitate separated, leaving a perfectly clear solution. The precipitate was washed several times by decantation with a 10 per cent. solution of ammonium sulphate. After this treatment the precipitate was collected upon a filter and thoroughly washed with water until entirely free from sulphates and then digested in alcohol and finally washed with alcohol and ether. The preparation thus

obtained was dried to constant weight at a temperature of 104°. Two samples of this proteid were prepared from different samples of flesh. Upon analysis the first sample (No. 110) gave 14.52 per cent. of nitrogen and 1.60 per cent. of sulphur. The second sample (No. 308*a*) contained 15.15 per cent. of nitrogen and 1.60 per cent. of sulphur. Hausmann's method as modified by Osborne and Harris gave the following results for the proportion of nitrogen in the different forms of combination occurring in the hydrolytic products.

TABLE XII.—PERCENTAGE OF NITROGEN IN THE DIFFERENT GROUPS, IN THE PROTEIDS SEPARATED UPON STANDING FROM A 10 PER CENT. AMMONIUM SULPHATE EXTRACT OF FLESH.

	First preparation. No. 110.	Second preparation. No. 308*b*.
Nitrogen as ammonia	1.16	1.19
Basic nitrogen	4.43	4.01
Non-basic nitrogen	8.65	9.33
Nitrogen in magnesium oxide precipitate	0.15	0.18
Nitrogen in the humin substances	0.10	0.14
Total nitrogen by summation	14.49	14.85
Total nitrogen in proteid	14.52	15.15

The clearly filtered ammonium sulphate extracts from which the above preparations separated upon standing are capable of undergoing fractional coagulation. A portion of one of the above ammonium sulphate extracts was maintained at a temperature of 50° for ten days. The coagulum formed was separated each day. A small quantity of coagulum would form within a few hours after each filtration. The coagula forming at 50° were combined and given laboratory No. 130. The filtrate obtained from the coagula separating at 50°, was kept at a temperature of 60° for a considerable time. A small coagulum formed which as yet has not been examined further. The filtrate from this coagulum at 60°, when heated to the boiling-point of water, gave relatively a considerable quantity of coagulated proteid which was separated, thoroughly washed with water, alcohol and ether and given laboratory No. 131.

Portions from each of the 10 per cent. ammonium sulphate extracts were completely coagulated at the temperature of boiling water, no fractions being separated at lower temperatures. The coagula were thoroughly washed with water and dilute alcohol until all sulphates were removed. They were then dried with

alcohol and ether and given laboratory Nos. 109 and 308*a*. These samples together with Nos. 130 and 131 mentioned above were dried to constant weight at a temperature of 104°, and the nitrogen was determined in each with the following results: No. 109, 15.44 per cent.; No. 308*a*, 15.83 per cent.; No. 130, 15.94 per cent.; and No. 131, 15.11 per cent. The sulphur in No. 109 equaled 2.25 per cent. and that in No. 308*a* amounted to 1.82 per cent.

The proportion of nitrogen in the different forms of combination that occur in the decomposition products produced by boiling preparations Nos. 109 and 308*a* with hydrochloric acid was determined.

TABLE XIII.—PERCENTAGE OF NITROGEN IN THE DIFFERENT GROUPS IN THE PROTEIDS SEPARATED UPON COMPLETE COAGULATION FROM A 10 PER CENT. AMMONIUM SULPHATE EXTRACT OF FLESH.

	First preparation. No. 109.	Second preparation. No. 308*a*.
Nitrogen as ammonia	1.01	0.92
Basic nitrogen	5.40	4.81
Non-basic nitrogen	8.88	9.66
Nitrogen in magnesium oxide precipitate	0.14	0.22
Nitrogen in the humin substances	0.01	0.04
Total nitrogen by summation	15.44	15.65
Total nitrogen in proteid	15.45	15.83

For the purpose of comparison a meat residue (laboratory No. 301) left after complete extraction with cold water and a residue left after complete extraction first with water and then with 10 per cent. ammonium sulphate solution (laboratory No. 307*a*) were hydrolyzed. The results are given in the following table.

TABLE XIV.—PERCENTAGE OF NITROGEN IN THE DIFFERENT GROUPS IN THE EXTRACTED RESIDUES OF FLESH.

	Residue. No. 301.	Residue. No. 307*a*.
Nitrogen as ammonia	1.25	1.08
Basic nitrogen	4.75	4.52
Non-basic nitrogen	10.01	10.05
Nitrogen in magnesium oxide precipitate	0.21	0.18
Nitrogen in the humin substances	0.07	0.05
Total nitrogen by summation	16.29	15.88
Total nitrogen in proteid	16.32	16.25

The flesh residues after the removal of the water extracts and the 10 per cent. ammonium sulphate extracts are readily freed

from the ammonium sulphate by washing with cold water. The washings so obtained contain only a mere trace of proteid matter. The washed residues after dehydrating with alcohol and ether are nearly white, having, however, as a rule a very slight grayish or yellowish tint. These residues closely resemble asbestos in general appearance. Two such residues have been analyzed for nitrogen and sulphur, and in one case the analogous analytical data were obtained for the residue after complete extraction with cold water but immediately before the extraction with the 10 per cent. solution of ammonium sulphate. The analytical data for the residue No. 203 which had been completely freed from the constituents soluble in cold water but which had not been extracted with the ammonium sulphate solution were as follows: Nitrogen 15.90 per cent., sulphur 1.05 per cent. and ash 0.70 per cent. The analogous data for the same original sample after complete extraction with a 10 per cent. ammonium sulphate solution, laboratory No. 214, were as follows: Nitrogen 15.88 per cent., sulphur 1.09 per cent. and ash 0.29 per cent.

A second residue representing the portion of flesh insoluble both in cold water and a 10 per cent. solution of ammonium sulphate (laboratory No. 307*a*) gave upon analysis 16.25 per cent. of nitrogen, 1.15 per cent. of sulphur and ash 0.39 per cent. The results of the hydrolysis of this same residue are given above, page 497.

This study of the proteids of flesh insoluble in cold water but soluble in a 10 per cent. solution of ammonium sulphate leads to the following conclusions:

First, that after flesh has been completely freed from proteids soluble in cold water, it contains two classes of proteid substances, namely, those which are soluble in a 10 per cent. solution of ammonium sulphate and those which are insoluble in this medium.

Second, that there could be separated from the turbid ammonium sulphate extract of flesh at least two individual proteids or groups of proteids which differ in physical properties and to some extent in chemical composition. These two substances or groups of substances differed from each other physically in that one of them, even if it was at first completely soluble in the 10 per cent. ammonium sulphate solution, which is doubtful, did not remain long in solution but separated from the same upon standing, while the other substance was readily and completely soluble in

this medium. These two proteid products separated from the turbid ammonium sulphate extract differ from each other in chemical composition. The one separating upon standing, containing a smaller percentage of total nitrogen (average 14.84), and sulphur (average 1.60) and also yielding upon hydrolysis less basic nitrogen (average 4.22) than the one which was separated by coagulation from the 10 per cent. ammonium sulphate extract. The analogous analytical data obtained for the latter substance were as follows: Total nitrogen 15.55 per cent., sulphur 2.04 per cent. and basic nitrogen 5.11 per cent. Of course these data regarding the composition of these two products must be considered more or less of a tentative nature until they are confirmed by further analyses.

Third, the percentage of sulphur in the two different proteid bodies obtained from the ammonium sulphate extract is very decidedly higher than it is in any of the other proteids which we have as yet separated from flesh. The highest sulphur content of any other proteid preparations of flesh so far obtained is 1.15 per cent. In the light of Osborne's work upon the vegetable proteids, it is probable that the higher content of sulphur in these products which are obtained from the ammonium sulphate solution is due to the fact that the solvent has reacted with the basic proteid molecule with the formation of a salt-like product. Further study is now being made in this connection to determine whether or not this is the case.

D. THE PROTEIDS OF FLESH INSOLUBLE IN COLD WATER, AND IN A 10 PER CENT. SOLUTION OF AMMONIUM SULPHATE, BUT SOLUBLE IN A COLD SOLUTION OF N/20 POTASSIUM HYDROXIDE.

The moist residues of flesh which remain after the complete extraction of fresh beef flesh, first, with cold water and second with a 10 per cent. solution of ammonium sulphate, when digested with a solution of N/20 potassium hydroxide, swell up at first to a semi-solid, jelly-like mass. Upon standing for several hours, most of the proteid apparently is dissolved but the resulting solution always remains turbid. It is practically impossible to filter the solutions through filter-paper. By straining them through cheese-cloth and through muslin the solid particles are removed and the extent of the turbidity is decreased, but a clear transparent solution is not obtained.

The N/20 potassium hydroxide solution of the flesh residue thus obtained may be treated with dilute hydrochloric acid until it is just neutral to phenolphthalein without causing a precipitation of the proteid. Again, the alkaline solution may be rapidly poured into an acid solution of such strength that the resultant solution is N/20 hydrochloric acid, without causing the precipitation of the proteid materials. When the alkaline solution is made distinctly acid to phenolphthalein the proteids separate as a white flocculent precipitate. If the proper quantity of hydrochloric acid is added, the liquid above the separated proteids becomes perfectly clear, the precipitate settles rapidly and it can be washed readily by decantation.

One residue of flesh which was left after extracting completely 10 pounds of lean beef round, successively with cold water, with a 10 per cent. solution of ammonium sulphate and with cold water to completely remove the last-mentioned solvent, was treated with a N/20 solution of potassium hydroxide. The resulting solution was filtered through muslin and precipitated by the addition of the proper amount of hydrochloric acid. The precipitate thus produced was washed thoroughly by decantation and dissolved in an N/80 solution of potassium hydroxide. This second solution of the proteid in the alkali was filtered through cheese-cloth and then reprecipitated with hydrochloric acid. The proteid thus precipitated was washed thoroughly by decantation and then upon a Büchner funnel by the use of a filter pump until the wash-water from the same gave no test for chlorides.

The large quantity of proteid obtained by the above treatment was divided approximately into six equal portions and each portion was further purified by the treatment indicated below.

Portion I was repeatedly extracted first with strong alcohol and then with ether. The proteid was air-dried and given laboratory No. 111.

Portion II was dissolved in a solution of N/80 potassium hydroxide and the solution resulting was filtered through cloth. It was then precipitated by the addition of the proper quantity of hydrochloric acid. The precipitated proteid was washed with water until entirely free from chlorides and then with alcohol and ether to remove fat and to properly air-dry the sample. The fraction of the proteid thus purified was labeled laboratory No. 112.

Portion III was twice subjected to the above treatment, that is, it was dissolved in the dilute alkali two times and precipitated by hydrochloric acid each time. The resulting product was thoroughly washed with water, alcohol and ether and given laboratory No. 113.

Portion IV was dissolved in an N/80 solution of potassium hydroxide and the solution produced filtered through cloth. The filtered solution was poured into a solution of hydrochloric acid of such a strength that the solution resulting would contain sufficient acid after the neutralization of the potassium hydroxide to form a N/40 solution of hydrochloric acid. The solution thus formed was not entirely clear but no precipitate separated from the same upon standing over night. The proteid contained in this acid solution was precipitated by the addition of a proper amount of potassium hydroxide. The precipitate was thoroughly washed with water, alcohol and ether and labeled No. 114.

Portion V was dissolved in potassium hydroxide and the solution thus formed poured into hydrochloric acid as described above for portion IV. The proteid contained in the acid solution in this case was, however, precipitated by the addition of one-fourth of its volume of a saturated solution of sodium chloride, containing enough of hydrochloric acid to make the acidity of the same equal to N/40. The resulting proteid precipitate was thoroughly washed with water by decantation and then dissolved in a solution of N/80 potassium hydroxide. This solution was strained through cheese-cloth and then precipitated by the addition of hydrochloric acid. The precipitate thus purified was washed very thoroughly with water, alcohol and ether. This preparation was labeled No. 115.

Portion VI was treated in the first place as portion V with the exception of the last operation. Instead of precipitating the alkaline solution directly with hydrochloric acid, it was poured into a solution of hydrochloric acid of such a strength that the solution resulting contained sufficient acid after the neutralization of the potassium hydroxide to form a N/40 solution of hydrochloric acid. The proteid dissolved in the excess of acid was precipitated by the addition of a solution of potassium hydroxide. The resulting precipitate was completely washed with water, alcohol and ether and then given laboratory No. 116.

TABLE XV.—CHEMICAL COMPOSITION OF DIFFERENT PREPARATIONS OF PROTEIDS OF FLESH INSOLUBLE IN WATER AND A 10 PER CENT. SOLUTION OF AMMONIUM SULPHATE BUT SOLUBLE IN A N/20 SOLUTION OF POTASSIUM HYDROXIDE.

Lab. No.	Method of purification.	Carbon. Per cent.	Hydrogen. Per cent.	Nitrogen. Per cent.	Sulphur. Per cent.	Ash. Per cent.
111	Dissolved two times in KOH, precipitated by HCl	51.90	7.37	15.82	1.14	0.52
112	Dissolved three times in KOH, precipitated by HCl	51.56	7.40	15.51	1.10	0.52
113	Dissolved four times in KOH, precipitated by HCl	51.56	7.43	15.26	1.11	0.55
114	Dissolved two times in KOH, poured into excess HCl, precipitated by KOH	51.26	7.38	15.45	1.09	0.50
115	Dissolved two times in KOH, poured into excess HCl, precipitated by NaCl, dissolved in KOH, precipitated by HCl	51.39	7.42	15.55	1.12	0.40
116	Dissolved two times in KOH, poured into excess HCl, precipitated by NaCl, dissolved in KOH, poured into excess HCl, precipitated by KOH	51.71	7.34	15.38	1.05	0.55
	Average	51.56	7.39	15.50	1.10	0.51

About 45 grams of air-dried proteid were obtained from each of the above treatments. Portions of each preparation were dried to constant weight at a temperature of 104° and then completely analyzed with the results obtained in the above table.

The analytical results of this experiment indicate clearly that the proteid material of flesh insoluble in water and in a 10 per cent. solution of ammonium sulphate, but soluble in a N/20 solution of potassium hydroxide solution, has the same chemical composition even when separated from the solvent by different means and purified by widely differing treatment.

While the results undoubtedly prove that we are dealing here with a comparatively pure proteid material, they do not by any means necessarily prove that the same is a single proteid substance, since it is often true that proteids differing greatly in physical properties and chemical behavior have practically the same chemical composition.

A composite sample was made of the preceding samples Nos. 111 to 116 inclusive and the resulting sample was carefully dried at 104°, and then hydrolyzed as usual with a 20 per cent. solution of hydrochloric acid. The results are given in the following table:

TABLE XVI.—PERCENTAGE OF NITROGEN IN THE DIFFERENT GROUPS, IN THE COMPOSITE SAMPLE (NOS. 111 TO 116, INCLUSIVE) OF THE PROTEIDS OF FLESH NOT SOLUBLE IN WATER OR AMMONIUM SULPHATE BUT SOLUBLE IN DILUTE POTASSIUM HYDROXIDE.

	Per cent.
Nitrogen as ammonia	0.98
Basic nitrogen	4.49
Non-basic nitrogen	9.92
Nitrogen in magnesium oxide precipitate	0.14
Nitrogen in the humin substances	0.07
Total nitrogen by summation	15.60
Total nitrogen in the proteid	15.50

A more detailed study of the decomposition products resulting upon the hydrolysis of the different proteid preparations obtained from the potassium hydroxide solution of the residue of flesh insoluble in water and in a 10 per cent. solution of ammonium sulphate is being made in this laboratory.

CONCLUSIONS.

(1) The total acidity of aqueous extracts of flesh varies between comparatively wide limits, the minimum being 0.66 per cent.,

the maximum 1.07 per cent. and the average 0.85 per cent., calculated as lactic acid and expressed in terms of the fresh substance of the meat.

(2) The residues of flesh which are left after the complete extraction with cold water are distinctly acid to litmus and to phenolphthalein.

(3) On the other hand, the insoluble proteids of flesh upon digestion with a N/10 solution of hydrochloric acid at the ordinary temperature combine with the latter, neutralizing its acid properties.

(4) Analyses made in this laboratory prove that 13.56 per cent. of the total proteid existing in lean beef flesh is soluble in cold water. Of this total soluble proteid 90.04 per cent. is in a form which is coagulable by heat from a neutral solution, 8.40 per cent. exists as albumoses and a very small quantity apparently exists in the form of peptones. It is not at present supposed that the albumoses and peptones exist as such in the original flesh.

(5) During the coagulation of an aqueous extract of flesh there is an increase in the acidity of the same. Reduction of the acidity of aqueous extracts of flesh facilitates coagulation of the proteids. One-fourth neutralization (to phenolphthalein) of the natural acidity of the cold water extracts gives such conditions in the resulting solutions, that the separation of the coagulable proteids is more complete than it is if further neutralization of the acidity is produced. The partial neutralization of the acidity of such extracts at the ordinary temperature causes the precipitation of some proteid matter.

(6) There are no well defined degrees of temperature at which different coagula of aqueous extracts of flesh separate. The complete removal of the proteid coagulating at any fixed definite temperature requires long application of heat. Further, the lower the temperature at which a coagulum is separated the longer the time of heating required to effect complete coagulation.

(7) Of the total protied of a cold water extract of flesh, that is, precipitated by neutralization and coagulation, 11.71 per cent. is separated by neutralization, 36.65 per cent. is precipitated by coagulation at a temperature below 50°, 39.93 per cent. coagulates between 51° to 75° and 11.71 per cent. is precipitated by coagulation at a temperature between 76° to 85°.

(8) The chemical composition of the different fractional coagual of the aqueous extracts of raw flesh is remarkably constant. They are also quite similar as regards their chemical constitution judging from the results of their hydrolysis.

(9) Raw flesh which has been completely freed from proteids soluble in cold water contains two classes of proteid substances, those which are soluble in a 10 per cent. solution of ammonium sulphate and those which are insoluble in this medium.

(10) Of the total proteids of lean raw flesh about 16.00 per cent. is insoluble in cold water but soluble in a 10 per cent. solution of ammonium sulphate.

(11) A 10 per cent. ammonium sulphate extract of flesh contains at least two individual proteids or groups of proteids which differ in physical properties and to some extent in chemical composition; one of them however may possibly be identical with the meat residue not dissolved by the ammonium sulphate.

(12) Raw flesh which has been completely freed from proteids soluble in cold water and also from those soluble in a 10 per cent. solution of ammonium sulphate is almost entirely soluble in a N/20 solution of potassium hydroxide.

(13) The proteid material of flesh insoluble in water and in a 10 per cent. solution of ammonium sulphate, but soluble in a N/20 solution of potassium hydroxide, has the same chemical composition even when separated from the solvent by different means and purified by widely differing treatment.

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